

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>MCA-448A PC</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/US 00/ 11926</b>	International filing date (day/month/year) <b>02/05/2000</b>	(Earliest) Priority Date (day/month/year) <b>04/05/1999</b>
Applicant <b>Millipore Corporation.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

contained in the international application in written form.

filed together with the international application in computer readable form.

furnished subsequently to this Authority in written form.

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the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2.  **Certain claims were found unsearchable** (See Box I).

3.  **Unity of Invention is lacking** (see Box II).

4. With regard to the **title**,

the text is approved as submitted by the applicant.

the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

the text is approved as submitted by the applicant.

the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

as suggested by the applicant.

because the applicant failed to suggest a figure.

because this figure better characterizes the invention.

1

None of the figures.



# INTERNATIONAL SEARCH REPORT

International Application No  
US 00/11926

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N15/10 C07K1/34 B01D61/14

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N C07K B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, FSTA, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 13963 A (HYMAN EDWARD D) 20 August 1992 (1992-08-20) * the whole document, in particular example 5, pages 15-16 and figures 1 and 4 * ---	1,8,10, 15,21
X	US 4 690 754 A (KOYAMA KENJI ET AL) 1 September 1987 (1987-09-01) the whole document ---	1,7,15, 17,19-21
X	WO 87 07645 A (LONDON HOSPITAL MED COLL) 17 December 1987 (1987-12-17) the whole document ---	1,7,15, 17,21
A	EP 0 431 905 A (TOSOH CORP ;RIKEN INST OF PHYSICAL AND CHE (JP)) 12 June 1991 (1991-06-12) the whole document --- -/-	1,2, 7-10,17

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance  
"E" earlier document but published on or after the international filing date  
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
"O" document referring to an oral disclosure, use, exhibition or other means  
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
"&" document member of the same patent family

Date of the actual completion of the international search

25 August 2000

Date of mailing of the international search report

12/09/2000

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H



## INTERNATIONAL SEARCH REPORT

International Application No

US 00/11926

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M.C. LINHARES AND P.T. KISSINGER: "Capillary ultrafiltration : in vivo sampling probes for small molecules" ANAL. CHEM., vol. 64, 1992, pages 2831-2835, XP000938635 the whole document -----	1, 3, 15, 17, 19-21



**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

[REDACTED] /US 00/11926

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9213963	A 20-08-1992	NONE		
US 4690754	A 01-09-1987	JP 61209010 A		17-09-1986
		DE 3608062 A		09-10-1986
		GB 2173711 A, B		22-10-1986
WO 8707645	A 17-12-1987	EP 0268647 A		01-06-1988
		JP 1500482 T		23-02-1989
EP 0431905	A 12-06-1991	JP 2978518 B		15-11-1999
		JP 3180182 A		06-08-1991
		DE 69008825 D		16-06-1994
		DE 69008825 T		25-08-1994



XP-000938635

# Capillary Ultrafiltration: In Vivo Sampling Probes for Small Molecules

Michael C. Linhares and Peter T. Kissinger\*

Purdue University, Department of Chemistry, West Lafayette, Indiana 47907

P.D. 1992  
P. 2831-2335 5

**Capillary ultrafiltration is a novel sampling method convenient for low molecular weight substances in living biological systems. By application of a negative pressure across a hydrophilic membrane capillary, small molecules are actively "pulled" across the membrane and collected. By elimination of large molecules and cellular matter, the ultrafiltrate collected is well suited for further analysis by liquid chromatography, capillary electrophoresis, or mass spectrometry. Ultrafiltration probes (UF probes) provide a simple means to obtain a small-volume sample from subcutaneous tissue, blood, saliva, or any other biological fluid in vivo. The dependence of recovery on flow rate, temperature, membrane dimensions, and vacuum magnitude are considered. The relative merits of capillary ultrafiltration probes and microdialysis probes are considered. UF probe applications presented include in vivo monitoring of drug disposition in human saliva and in the subcutaneous space of awake, freely moving rats.**

## INTRODUCTION

Determination of low molecular weight substances in fully functioning biological systems is a goal long sought by scientists in pharmacology, toxicology, drug metabolism, and clinical chemistry, among other disciplines. A great deal of effort is directed at obtaining reproducible samples rapidly enough to follow the metabolic events of interest. In addition, removing undesirable components that might interfere with the determination of the analyte of interest is necessary. Although the ideal methodology does not exist at this time, microdialysis<sup>1-5</sup> and ultrafiltration<sup>6,7</sup> show great promise for in vivo monitoring.

Ultrafiltration has been used in vitro for sample preparation by means of centrifugal membrane filters for several decades. Capillary ultrafiltration probes provide a new format to address several of the problems associated with sampling in biological media. The concept was originally developed for in vivo use specifically as a part of a glucose monitoring system for diabetics.<sup>8</sup> Capillary ultrafiltration probes (UF probes) use a negative pressure gradient to create a flow of fluid across a hydrophilic membrane. The membrane excludes proteins and other cellular matter while allowing the extracellular fluid and small molecules or ions to pass through. This provides an ultrafiltrate that is well suited for analysis by liquid chromatography, capillary electrophoresis, or mass spectrometry. Since the process of ultrafiltration is a convective

process, high relative recoveries are achieved for small analytes, typically greater than 90%. UF probes can be implanted in vivo in subcutaneous tissue for long periods of time, at least 6 months,<sup>9</sup> and provide clean samples rapidly (1-5  $\mu$ L/min). The magnitude of analyte flux across the membrane is a function of temperature, pressure differential, membrane dimensions and material. A preliminary study of these parameters and the other practical factors affecting the use and implementation of UF probes for in vivo study are presented here.

It is beneficial to consider the differences and similarities of capillary ultrafiltration and capillary microdialysis for the sampling of small molecules in vivo. Microdialysis has proven to be an excellent sampling technique for in vivo sampling of the brain, liver, eye, and heart and subcutaneous area. It shows great promise for studies of the metabolism, pharmacokinetics, and bioavailability of therapeutic drugs.<sup>1-5</sup>

Due to their simplicity and ruggedness, UF probes have the potential to be used for many applications. Two initial areas of investigation were explored in this study. In vivo saliva collection is one area of interest. Saliva has been shown to be a good media to monitor some therapeutic drugs<sup>8-10</sup> and metabolites,<sup>11</sup> and has been shown to track the concentration of many small molecules in blood plasma quite well. The disposition of controlled release theophylline tablets was monitored in saliva by capillary ultrafiltration. Monitoring of therapeutic drug disposition in subcutaneous tissue is also well suited to study using the capillary ultrafiltration technique. The disposition of acetaminophen was observed in awake freely moving rats.

## PRINCIPLES OF ULTRAFILTRATION

In ultrafiltration, a pressure gradient across a semipermeable membrane causes solvent (water) and small analytes to pass through the membrane leaving large substances behind. The flux ( $\text{mol}/(\text{cm}^2 \text{ s})$ ) through the membrane is dependent on the size of the given analyte, the transmembrane pressure, the viscosity of the fluid (sample media), the membrane thickness, and the structure of the membrane. The flux dependence on the pressure gradient is nonlinear at lower vacuum gradients and becomes constant at higher gradients. The mathematical relationships and other features of the ultrafiltration process have been reviewed.<sup>12</sup> The influence of temperature is very critical during in vivo experiments in that membrane fluidity, solution viscosity, blood flow, and metabolic rates are all affected. Polyacrylonitrile membranes with reported molecular weight cutoffs (MWCO) of 30 000 were used in this study. Since the

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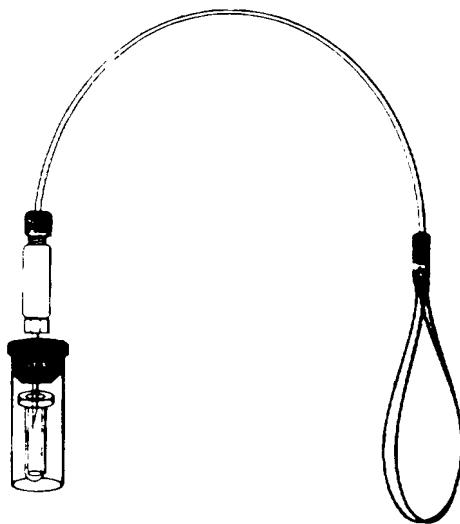


Figure 1. Schematic of a UF-3-7 PAN capillary ultrafiltration probe with a Vacutainer collection system.

membrane is under a vacuum, thick membranes, typically  $>40\text{ }\mu\text{m}$  of small internal diameter are more successful, in that the membrane does not collapse under the applied vacuum. Thicker membranes do, however, lower the flux and the recovery of some analytes due to interactions with the membrane structure. The UF probes utilized here have three membrane loops of 7–16 cm and provide flow rates of 2–5  $\mu\text{L}/\text{min}$ . These are not intended to be the ultimate embellishment of this technique. Other formats are being utilized for other specific applications.

## EXPERIMENTAL SECTION

**Ultrafiltration Probes.** Prototype ultrafiltration probes UF-3-7, UF-3-12, and UF-3-16 were provided by Bioanalytical Systems, BAS (West Lafayette, IN). These probes consisted of three loops of polyacrylonitrile (PAN) membrane between 7 and 16 cm long. The PAN membrane is 310- $\mu\text{m}$  o.d. and 220- $\mu\text{m}$  i.d. and has a reported 30 000 MWCO. A 1-cm length of the implanted membrane filter has an internal volume of 0.38  $\mu\text{L}$ . The probes use Teflon tubing for connection to a vacuum source and use soft polyurethane connectors to allow for greater biocompatibility and flexibility.<sup>13</sup> An example of a capillary ultrafiltration probe, with a Vacutainer collection vessel, is shown in Figure 1.

**Vacuum Sources.** Two primary methods were used to create the vacuum for ultrafiltration. A 2-mL Vacutainer (Becton/Dickinson, Rutherford, NJ) with approximately 1-in. Hg works very well for collecting samples. A small 250- $\mu\text{L}$  vial (Chromacol, 03-CVG, Trumbull, CT) is placed inside the Vacutainer, and the vacuum is replaced by using a 60-mL syringe. An example is shown in Figure 1. The second method is the use of a peristaltic pump. A peristaltic pump (Minipuls 2, Gilson, France) using 0.007-in. i.d. tubing can produce a variable but controlled flow which supports a pressure differential. The peristaltic pump is useful when it is desirable for the collection of samples to be continuously automated. A modified peristaltic pump tubing with 100- $\mu\text{m}$ -i.d. fused-silica tubing inserted inside has provided an excellent system for automated collection with an internal volume less than 7  $\mu\text{L}$ . Coupled to a CMA/140 fraction collector (BAS, West Lafayette, IN) it permits totally unattended operation of sample collection. The peristaltic pump was used for characterization and in vivo subcutaneous tissue studies, while the Vacutainer system was used only for in vivo saliva collection studies.

**Ultrafiltration Characterization.** A Metler AE 166 balance was used for weighing the ultrafiltrate samples during bulk flow

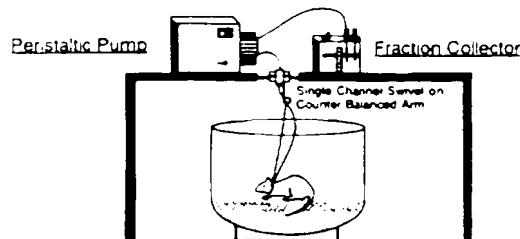


Figure 2. Schematic of the awake animal capillary ultrafiltration collection system.

determinations. Samples were collected for a specific time, usually 5 min, and weighed. Timing was done using a stopwatch. For temperature control a Pierce Reacti-therm (Rockford, IL) heating block was used. All in vitro solutions were constantly stirred using a magnetic stirring plate.

**Monitoring of Theophylline in Saliva.** Experimental subjects fasted 12 h before the experiment and 2 h after dosing (Uniphyl 400-mg 24-h controlled release tablet) with 180 mL of water. After flushing the mouth with water and chewing on a piece of parafilm for about 1 min to stimulate saliva flow, samples were taken for 3 min, at 15-min intervals for 4 h. Samples were then directly analyzed by reversed-phase liquid chromatography.

**In Vivo Monitoring of Acetaminophen in Subcutaneous Tissue.** Long Evans rats (200–250 g) were anesthetized using ketamine/xylazine (0.1 mL/100 g). Either a UF-3-12 or UF-3-16 probe was implanted in the subcutaneous tissue of the back. The probe was sutured to the skin to secure it. The rats were placed in an BAS/CMA awake animal bowl. A collar was attached and secured through a wire theather to a single channel swivel attached to a counter-balanced arm. This arrangement allows for freedom of movement for the animal and is illustrated in Figure 2. After the rats recovered from surgery, between 8 and 12 h, the UF probe was attached to a peristaltic pump through the swivel. The pump was set to a flow rate of 44  $\mu\text{L}/\text{min}$ ; producing a vacuum to achieve an ultrafiltration rate of  $2.4 \pm 0.1\text{ }\mu\text{L}/\text{min}$  ( $n = 56$ ). A stock solution of acetaminophen (1 mg/mL) was made in Ringer's solution, and 2.0 mg/kg was injected interperitoneally. Ultrafiltrates were collected continuously using a BAS/CMA 140 fraction collector and were directly analyzed by reversed-phase liquid chromatography.

**Chromatography.** Liquid chromatography was carried out with a BAS 200A liquid chromatograph (Bioanalytical Systems, West Lafayette, IN). Separations were carried out on a Biophase C<sub>18</sub>, 3- $\mu\text{m}$ , 100-mm by 3.2-mm column at 35 °C and 1 mL/min, except for subcutaneous acetaminophen determination which used a Biophase 250-mm by 4.6-mm octylsilane column. For the separation of theophylline in saliva a mobile phase of 100 mM phosphate buffer at pH 6.1 with 5% acetonitrile was used. Detection for theophylline was accomplished using the variable-wavelength UV detector set at 270 nm. For acetaminophen determinations the mobile phase consisted of 75 mM monochloroacetic acid (pH 3.1), 0.67 mM EDTA, and 2.5% acetonitrile was used. Electrochemical detection was accomplished using a 3-mm glassy-carbon electrode at +750 mV vs Ag/AgCl.

**Capillary Electrophoresis.** An in-house-built capillary electrophoresis system was used for all experiments. Brij 35/alkylsilane capillaries prepared according to the method of Towns and Regnier<sup>14</sup> were used for separations. The 75- $\mu\text{m}$ -i.d. capillaries were 50 cm long, with detection at 25 cm. Detection was accomplished by UV absorption at 214 nm. Injections were done by hydrodynamic syphon for 10 s at 20 cm. Samples were electrophoresed under a potential of 300 V/cm.

**Reagents.** Acetaminophen, theophylline, and protein standards were purchased from Sigma (St. Louis, MO) and used as received. All buffer reagents were reagent grade and purchased from Aldrich Chemical (Milwaukee, WI). HPLC grade acetonitrile (Baxter) was used. All solutions were made with double distilled deionized water and filtered using 0.22- $\mu\text{m}$  Nylon filters.

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Table I. Comparison of Ultrafiltration and Microdialysis as in Vivo Sampling Methods

	microdialysis	ultrafiltration
sample condition	physiological saline solution-ready for analysis	extracellular fluid-ready for analysis
sample dilution	yes, 5-30%	relatively little, >90%
spatial resolution	conc recovery	conc recovery
time resolution (typical)	excellent	good in specific tissue
	1-5 min	1-5 min
	(both depend on the sample volume needed)	
flow rate control	yes, very precise	limited $\pm 5\%$
sample collection rate	0.1-10 $\mu\text{L}/\text{min}$	0.1-10 $\mu\text{L}/\text{min}$
mol wt range	recoveries drop exponentially with increasing mass	depends on membrane structure and compound of interest
mode of collection	passive-diffusion conc gradient	active-pressure gradient
awake animals	yes	yes
tissue restrictions	none	yes, requires large fluid turn over
ease of automation	fraction collection and on-line to instrument	fraction collection only

## RESULTS AND DISCUSSION

Sampling and determining the extracellular concentration of small molecules in living biological systems present a huge problem. No single methodology is useful for all situations. Capillary ultrafiltration probes provide a solution to the problems of time-dependent sampling, rapid continuous sampling, enzymatic degradation of the sample, and sampling freely behaving animals. In addition to capillary ultrafiltration, microdialysis also provides these advantages.<sup>1-5</sup> Low relative recoveries due to sample dilution, delicate probes with rigid components, and uncertainties in calculating interstitial concentrations can present hurdles for microdialysis in some circumstances.<sup>15</sup> Capillary ultrafiltration may provide a solution to several of these concerns. Table I outlines the differences and similarities of these two in vivo sampling techniques.

UF probes provide a sample in which all high molecular weight components including enzymes, proteins, and cellular particulate matter are removed. In order to observe the effectiveness of capillary ultrafiltration to remove high molecular weight molecules, samples of proteins with varied molecular masses were ultrafiltrated and analyzed by capillary electrophoresis. The proteins included apoprotein (MW 6500), insulin (MW 5800), myoglobin, cytochrome c, ribonuclease, and serum albumin. Using the PAN membranes, no protein was ever seen in the ultrafiltrate. These results lead us to believe that the PAN membranes effective MWCO is much lower than 30 000 when used under a negative pressure and they are effective at removing any potentially interfering proteins.

Capillary ultrafiltration probes provide very high recoveries of small molecules. One concern with microdialysis is that very low relative concentration recoveries are achieved, since it is a diffusion-based technique using small probes. In vitro and in vivo recoveries are seldom identical due to the fact that diffusion of the analytes through the tissue is the limiting factor in the collection of the analyte.<sup>16</sup> Typical microdialysis recoveries are between 5 and 30% at flow rates of 1  $\mu\text{L}/\text{min}$ . Ultrafiltration provides higher recoveries, typically greater than 90%, because the process includes an active transport of fluid across the membrane. Table II illustrates recoveries of several different small molecules of interest. The membrane only acts by a sieving mechanism and the pressure difference creates the flux across the membrane (rather than diffusion alone). Ideal (100%) recoveries are not obtained for all molecules. Water molecules and small ions are

Table II. Ultrafiltrate Recovery for Several Compounds of Interest

compd	% recovery $\pm \text{SD}^a$	compd	% recovery $\pm \text{SD}^a$
acetaminophen	97.6 $\pm$ 0.83	ascorbic acid	99.5 $\pm$ 1.05
cefazolin	95.2 $\pm$ 1.83	glucose	98.0 $\pm$ 1.00
theophylline	100.2 $\pm$ 0.80	amoxicillin	97.3 $\pm$ 1.36
tryptophan	92.7 $\pm$ 0.095		

<sup>a</sup>  $n = 5$  for all analytes. All samples were prepared in Ringer's solution, and experiments were performed at 27 °C with constant stirring. Each analyte concentration was between 1 and 5  $\mu\text{g}/\text{mL}$ .

Table III. Comparison of Ultrafiltration Recoveries with Variation in Concentration of Analyte

conc of acetaminophen, $\mu\text{g}/\text{mL}$	% recovery $\pm \text{SD}^a$	conc of acetaminophen, $\mu\text{g}/\text{mL}$	% recovery $\pm \text{SD}^a$
10	99.7 $\pm$ 0.90	0.5	100.7 $\pm$ 1.41
5	100.7 $\pm$ 1.3	0.25	100.4 $\pm$ 2.3
1	98.6 $\pm$ 1.27		

<sup>a</sup>  $n = 5$  for all concentrations. All samples were prepared in Ringer's solution, constantly stirred, and performed at 27 °C.

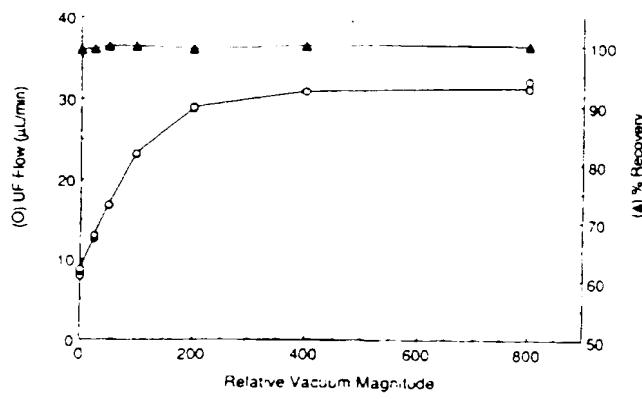
statistically more likely to pass through the membrane unobstructed and are less likely to experience hydrophobic or electrostatic interactions with the membrane. As a consequence recoveries are typically greater than 90% for small molecules. Variation in concentration of a analyte does not change the relative recovery. Table III illustrates recoveries for acetaminophen at several different concentrations. Using the students *t* test, these values are not statistically different.

Capillary ultrafiltration can be used for in vivo sampling in awake animals. Ultrafiltration probes are well suited for use in tissues that have a high fluid turnover and are not substantially affected by loss of fluid. A typical ultrafiltration capillary removes extracellular fluid at 50 ( $\text{nL}/\text{min}$ )/mm of membrane. UF probes can provide constant sampling rates in subcutaneous tissue. Using UF-3-16 PAN a flow rate of  $2.4 \pm 0.1 \mu\text{L}/\text{min}$  ( $n = 56$ ) was obtained in subcutaneous tissue. Only tissue that can sustain these losses are potential sampling sites for ultrafiltration. Microdialysis probes are especially useful for small animals and organs. There is no net fluid loss from the tissue in microdialysis, and this is especially advantageous in organs that cannot sustain fluid loss. The effects of loss of extracellular fluid in relation to physiological response is unknown at this time.

UF probes are currently quite large compared to most microdialysis probes. Using polyacrylonitrile membrane material, fibers need to be at least 7-16 cm long to achieve

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**Figure 3.** Ultrafiltrate flow (O) for a UF-3-7 PAN probe and relative recovery (▲) of acetaminophen at increasing vacuum (as indicated by nominal flow rate setting on a peristaltic pump).

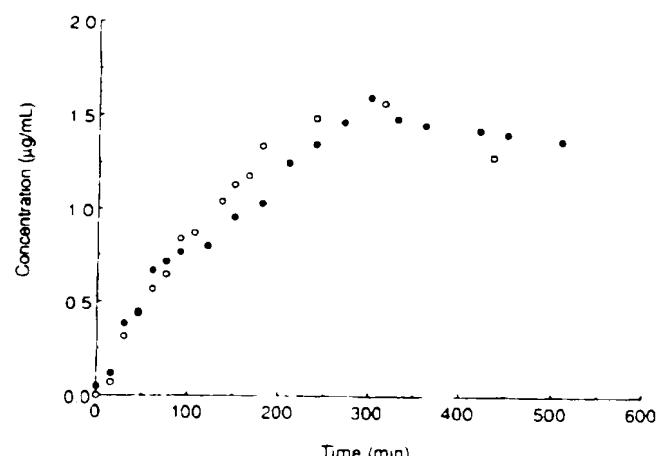
practical *in vivo* flow rates ( $1-5 \mu\text{L}/\text{min}$ ), whereas microdialysis capillaries under 1 cm in length are quite common. Since UF probes remove extracellular fluid from the animal, the temperature and activity of the animal are important. In anesthetized animals the drop in body temperature and circulation of fluids can cause decreased ultrafiltration rates. The ultrafiltration flow has been found to change linearly *in vitro* at a rate of  $0.3 (\mu\text{L}/\text{min})/\text{°C}$  for a UF-3-7 probe ( $n = 3$ ).

In ultrafiltration the flow rate is dependent on the dimensions of the membrane (membrane surface area) and differential pressure, while relative recovery is not. This is illustrated in Figure 3, which shows both the dependence of UF flow on vacuum magnitude for a UF-3-7 PAN probe and the constant recovery of acetaminophen obtained at these vacuum magnitudes. As it is illustrated, the UF flow is dependent on vacuum magnitude but is limited by the flux of water across the membrane. The probe size or membrane surface area and vacuum magnitude are the factors that limit UF flow. If more rapid sample collection rates are required, a larger membrane surface area can be used. In microdialysis the relative recovery is exponentially related to the perfusion flow rate since the transport is mediated by diffusion. The sampling rate is therefore limited by the volume of sample needed for analysis. Most microdialysis experiments are carried out at flow rates less than  $3 \mu\text{L}/\text{min}$  for this reason.

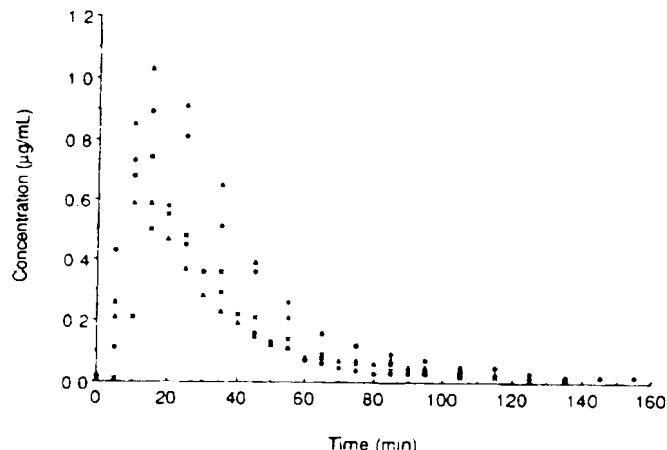
Capillary ultrafiltration can be carried out continuously *in vivo* in awake animals for extended periods of time with fully automated systems. Microdialysis has become very popular due to the ability to sample awake animals continuously and conduct lengthy experiments with either on-line analysis or automated sample collection systems. Capillary ultrafiltration probes can function similarly. UF probes implanted in subcutaneous tissue can be attached to a peristaltic pump and fraction collector, enabling samples to be automatically collected. Ultrafiltration can be more difficult with on-line chromatographic systems due to "outgassing" (bubble segmented flow in the collection line).

**Therapeutic Drug Monitoring in Saliva.** Saliva is a biological fluid that has great promise as a therapeutic drug monitoring medium.<sup>8-10</sup> For many drugs, metabolites, and biomolecules the saliva concentrations are a good representation of blood plasma levels.<sup>9</sup> Ultrafiltration probes are well-suited for sampling in saliva. The UF-3-7 PAN probes provide a rapid *in vivo* sampling method that is not invasive, avoids the unpleasantness of expectorating into a cup, and can provide  $20 \mu\text{L}$  of "clean" microbe and protein-free sample in 4 min. This allows for more simplified assay methods and direct injection of the ultrafiltrate.

To demonstrate this concept, the monitoring of the common bronchial dilator theophylline in saliva is presented. A



**Figure 4.** Two examples of *in situ* monitoring of theophylline in saliva after dosing with a controlled release 400 mg of Uniphy tablet using capillary ultrafiltration. Both curves were done on the same subject on separate days.



**Figure 5.** Disposition curves for five different rats dosed with  $2.0 \text{ mg/kg}$  acetaminophen obtained using capillary ultrafiltration probes implanted in the subcutaneous tissue.

pharmacokinetic model for theophylline using saliva as a monitoring medium has been reported.<sup>17</sup> Using UF-3-7 PAN probes theophylline concentration was followed in saliva. Two representative disposition curves are presented in Figure 4. This was only a preliminary study to explore the possible utility of UF probes, not to determine pharmacokinetic parameters for the dosage form used.

**In Vivo Monitoring of Acetaminophen.** The monitoring of the disposition of APAP in subcutaneous tissue in awake rats is presented. Ultrafiltration probes were implanted into the subcutaneous tissue along the back of five rats for these experiments. The animals were anesthetized before implantation and then allowed to recover for 8–12 h prior to dosing interperitoneally (2.0 mg/kg). The dynamics of acetaminophen was monitored while the animals were awake and freely moving. The results from five different animals are shown in Figure 5. These results compare very well with microdialysis experiments monitoring acetaminophen in the jugular vein of rats.<sup>5</sup>

## CONCLUSIONS

Capillary ultrafiltration probes provide an alternative sampling technique for small molecules in living biological

systems. UF probes remove large molecules, enzymes, and cellular matter that can degrade analytes and instrument performance, while providing high recoveries of small molecules. UF probes are made from biocompatible, soft components and can be implanted in awake animals for extended periods. UF probes are ideal for monitoring drug disposition in subcutaneous tissue and can be used *in situ* for saliva collection. In addition to the experiments presented here the dynamics of cefazolin, amoxicillin, theophylline, glucose, lactic acid, and pyruvate have been monitored in

*vivo* by ultrafiltration and will be presented in future publications.

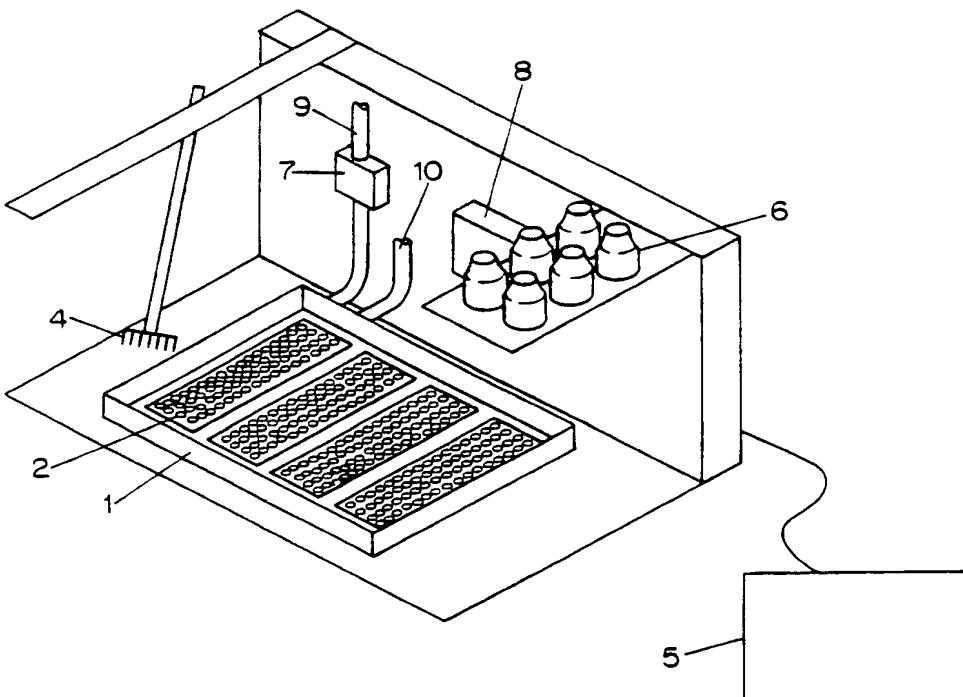
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(71)(72) Applicant and Inventor: HYMAN, Edward, D. [US/US]; 2100 Sawmill Road, River Ridge, LA 70123 (US).					
(74) Agents: NEUNER, Robert et al.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112 (US).					
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(54) Title: METHOD FOR PREPARATION OF CLOSED CIRCULAR DNA					
(57) Abstract					
<p>Closed circular DNA can be recovered from a cells or subcellular organelles containing closed circular DNA by a method comprising, in sequence, the steps of: (a) lysing the cells or organelles to release DNA, for example with a combination of lysozyme and heat or detergent; (b) enzymatically treating the released plasmid DNA with a proteolytic enzyme such as Proteinase K; (c) enzymatically treating the proteolytic enzyme-treated plasmid DNA with RNase and topoisomerase (I); (d) selectively enzymatically digesting the non-circular nucleic acids from the microorganism; and (e) recovering closed circular DNA. The method is particularly applicable to recovery and purification of plasmid DNA from <i>E. coli</i>.</p>					
					

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DescriptionMethod For Preparation of Closed Circular DNABackground of the Invention

This application relates to a new method for preparing plasmid and other closed circular DNA which is readily automated.

Plasmids are small circular pieces of DNA found naturally and as a result of human intervention in many bacteria, particularly E. coli. Because many copies of an individual plasmid are frequently present within the host organism, and because plasmids are both reproduced and in many cases expressed by the host organism, plasmids have come to play a major role in many biotechnological processes. Indeed, the preparation of plasmid DNA containing a nucleic acid fragment of interest is a routine procedural precursor to such other procedures as DNA sequencing, restriction digestion, cloning, probing, amplification (e.g., PCR), hybridization, in vitro transcription or mutagenesis.

At present, there are two methods which are commonly used to prepare plasmid DNA from cultures of E. coli: the alkaline lysis method and the boiling method. Sambrook et al., Molecular Cloning, 2d ed., p. 1.21 (1989). Both methods depend on the use of centrifugation to purify plasmid at several stages of the procedures, including centrifugation of chromosomal DNA, centrifugation of phenol and chloroform extractions, and centrifugation of the ethanol precipitate. In the alkaline lysis method, sodium hydroxide and sodium dodecyl sulfate (SDS) are used to lyse cells, after which acetate and acetic acid are added to precipitate chromosomal DNA. The precipitated DNA is removed by centrifugation and the supernatant is recovered and extracted with 1:1 phenol:chloroform.

Plasmid DNA is then precipitated by adding ethanol and recovered by centrifugation. Finally, RNA is removed from the recovered plasmid DNA by resuspending the material in buffer containing pancreatic RNase.

5        The boiling method for preparing plasmids uses lysozyme in a boiling water bath to lyse the cells. Chromosomal DNA and cellular debris are removed by centrifugation and the supernatant containing the plasmid DNA is treated with sodium acetate and 10      isopropanol to precipitate the plasmids. This precipitate is recovered by centrifugation, and treated with RNase.

15      One commercially available instrument for preparing plasmid DNA contains a built in centrifuge and isolates plasmid DNA using the alkaline lysis method. The throughput of this machine is limited to about 40 plasmid preps per day, the equivalent of what a single technician can achieve by hand in one day. 20      The human genome project and other efforts which could involve the need for hundreds of thousands of plasmid preparations before completion make it clear that this throughput level is unacceptable. There exists, therefore, a real need for a preparation technique which can be performed rapidly and simply by automated 25      equipment. This invention satisfies that need.

#### Summary of the Invention

In accordance with an embodiment of the invention, closed circular DNA can be recovered from cells or 30      subcellular organelles containing closed circular DNA by a method comprising, in sequence, the steps of:

(a) lysing the cells or organelles to release DNA, for example with a combination of lysozyme and heat or detergent;

- (b) enzymatically treating the released plasmid DNA with a proteolytic enzyme such as Proteinase K;
- (c) enzymatically treating the proteolytic enzyme-treated plasmid DNA with RNase and topoisomerase I;
- (d) selectively enzymatically digesting the non-circular nucleic acids from the microorganism; and
- (e) recovering closed circular DNA.

10 The method is particularly applicable to recovery and purification of plasmid DNA from E. coli.

#### Brief Description of the Drawings

Fig. 1 shows an automated instrument for 15 performing the method of the invention;

Fig. 2 shows a modified microtiter sample holder useful in automating the present invention.

Figs. 3 and 4 show cross sections of a single sample well.

20

#### Detailed Description of the Invention

The present invention is applicable generally to the isolation of closed circular DNA, both single and double stranded, from other DNA. Thus, the invention 25 is applicable both to the preparation of plasmid samples and to preparation of circular phage DNA (e.g. phage M13 which is frequently used as a cloning vector) and DNA isolated from organelles such as chloroplasts and mitochondria. The method can also be employed in 30 modified form to isolate viral DNA from host nucleic acids.

The first step of the method for preparing purified circular DNA in accordance with the invention is lysis of the host microorganism. For E. coli, lysis 35 can be accomplished by a combination of enzymatic treatment with lysozyme followed by heat treatment at a

time and temperature sufficient to accomplish the result of making DNA available for subsequent enzymatic attack. Other methods for cell lysis can also be employed provided that the lysis method does not nick 5 the plasmid DNA. For example, exposure to alkaline conditions (pH 13-14) after lysozyme treatment or species specific-lytic agents such as lysostaphin for staphylococci may be employed. Lyticase or zymolase can be used to lyse yeast cells, while detergent alone 10 should be sufficient in the case of mammalian cells, insect cells (e.g. for baculovirus isolation), chloroplasts or mitochondria. Cellulase may be a suitable lytic enzyme for isolation from plant cells.

Following cell lysis, the cell preparation is 15 enzymatically treated with a protease enzyme to destroy the cell structure and free nucleic acids from proteins (e.g., histones) which might interfere with subsequent enzymatic degradation steps. A suitable enzyme for this purpose is Proteinase K, a commercially available 20 protease secreted extracellularly by Tritirachium album (Sigma Chemical Co., St. Louis, MO). Proteinase K is a serine endoprotease of the bacterial subtilisin family that rapidly and non-specifically hydrolyzes native proteins. Conveniently, proteinase K remains 25 active in the presence of urea and SDS and other detergents such that denaturants may be added to facilitate the proteolytic digestion and is active at relatively high temperatures which allows accelerated proteolysis. Detergents, such as TRITON X-100, may 30 also be added to facilitate the proteolytic digestion.

After incubating the lysed bacteria in the protease enzyme for a period of time sufficient to substantially clarify the preparation, the protease 35 enzyme is inactivated to prevent degradation of subsequently added enzymes. This inactivation is conveniently done by heating the preparation, which not

only inactivates the protease but also denatures substantially all of the bacterial DNA other than supercoiled and relaxed closed plasmid DNA into single stranded form. Other inactivators may be used (e.g., 5 metal chelators, phenylmethylsulfonyl fluoride, iodoacetate or a change in pH (increasing or decreasing from optimum as appropriate for the enzyme used) or buffer conditions), however if heat inactivation is ineffective for a given protease, provided that these 10 inactivators do not interfere with subsequent enzymatic steps (e.g., nuclease activity) or can be removed. In addition, chemical means for denaturing non-circular DNA may be employed, including the enzyme helicase, exposure to alkali (pH 13-14), acid (pH 1-4), urea, 15 dimethylsulfoxide or dimethylformamide or other denaturing conditions.

Upon cooling the solution, the chromosomal DNA is likely to remain in single stranded form as a result of the low incidence of highly repetitive sequences in 20 bacterial, e.g. E. coli, genomes. Thus, the chromosomal DNA, along with the RNA, is susceptible to enzymatic digestion by a variety of nucleases, including single stranded exo- and endonucleases and double stranded exonucleases. Suitable nuclease 25 enzymes for this purpose include Mung Bean Nuclease, S1 Nuclease (US Biochemical Corp., "USB"), P1 Nuclease (Bethesda Research Labs, "BRL"), T7 exonuclease (USB), Bal 31 Nuclease (USB), Exonuclease I (USB), Exonuclease III (USB), Exonuclease VII (BRL) and Lambda Exonuclease (BRL). RNA from the microorganism is digested either 30 before or concurrently with the DNA, depending on the conditions needed for enzymatic activity, using an RNase enzyme such as pancreatic ribonuclease (USB) or Ribonuclease T<sub>1</sub> (Pharmacia).

35 In selecting a nuclease for use in the present method, it is important to remember that some nuclease

enzymes, notably S1 nuclease and Bal 31 nuclease digest supercoiled plasmid DNA as well as single stranded DNA. For example, Bal 31 nuclease degrades single stranded DNA endonucleolytically and it hydrolyzes both strands 5 at the end of double stranded DNA. Bal 31 nuclease will completely hydrolyze supercoiled plasmid DNA due to the fact that supercoiled plasmid exists transiently in single stranded form, which is a substrate for Bal 31. Legerski et al., *J. Biol. Chem.* **252**, 8740 (1977).

10 To avoid unwanted digestion of plasmid DNA, enzymes of this type should be used after treatment with an enzyme such as topoisomerase I (BRL) which relaxes supercoiled plasmid DNA. Other enzymes which would achieve this same result would be a combination of DNase I

15 (nickase), DNA ligase and ATP/NAD; or T4 DNA ligase and AMP. Both of these combinations would nick supercoiled DNA to yield a nicked, relaxed plasmid, and then repair the nick via the ligase. Relaxation of supercoiled DNA can also be achieved non-enzymatically with

20 intercalating agents such as ethidium bromide.

On the other hand, T7 exonuclease degrades the 5' end strand of double stranded DNA attacking only chromosomal DNA which exists as linear double strands. Ausubel et al., eds, *Current Protocols in Molecular 25 Biology*, p. 3.12.1. T7 exonuclease will not degrade either supercoiled or relaxed plasmid. In fact, the resistance of plasmids to degradation serves as a quality control test for T7 exonuclease purity used by the manufacturer (USB).

30 Other options for selective removal of chromosomal DNA include the use of DNA polymerase enzymes, which under certain conditions (absence of dNTP's and excess pyrophosphate, i.e., about 100mM) catalyze the reverse polymerization of double stranded nucleic acids. This 35 activity requires a free 3'-OH group and thus is specific for linear (non-plasmid) DNA. These enzymes

also have exonuclease activity for either double or single stranded DNA. Kornberg, DNA Replication, W.H. Freeman & Co., pp 127-130 (1980). Suitable enzymes include Klenow fragment, T7 DNA polymerase (USB), Taq 5 polymerase and T4 DNA polymerase.

Additional enzymes might be used in conjunction with a reverse polymerization system to enhance the rate of degradation. These enzymes include T4 polynucleotidokinase to remove the 3'-terminal 10 phosphate and to yield a 3'-OH group, enzymes selected to degrade the dNTP product, such as hexokinase and nucleoside diphosphate kinase together with appropriate reagents (ADP and glucose).

Terminal deoxynucleotidyl transferase (TdT) could 15 also be theoretically used to achieve selective degradation of single stranded chromosomal DNA in the presence of pyrophosphate. Like DNA polymerases, this enzyme requires a free 3'-OH group, but it acts on single stranded as opposed to double stranded nucleic 20 acids. The dNTP's released could be further hydrolyzed with the aid of the same enzyme systems discussed above. This system is not a preferred means for selective single stranded DNA removal, however, as the reaction rate is very slow.

25       Restriction enzymes are potentially useful in the present invention provided the plasmid to be isolated does not have the restriction site for which the enzyme is specific. A simple test can be used to assess the presence or absence of a given restriction site in a 30 plasmid by exposing the plasmid preparation to the restriction enzyme prior to heat treatment and digestion of chromosomal DNA and running a gel to see if plasmid DNA remains. If plasmid DNA is observed, the restriction site is not present in the plasmid. If 35 restriction enzymes are used it is appropriate to maintain chromosomal DNA in a double stranded state

until restriction digestion has occurred. This may require lowering the temperature used to denature the protease.

5 If the product desired is single stranded circular DNA, nucleases with single-strand endonuclease activity cannot be used as these enzymes will degrade the desired product. Thus, in this case, a combination of nucleases is selected from among single-strand exonucleases and double-strand exonucleases. Double-  
10 strand endonucleases can also be employed provided that the circular DNA does not self anneal to produce double stranded regions or if conditions (temperature/buffer) are maintained to prevent formation of double stranded regions.

15 The final step in the nucleic acid preparation of the present invention is the recovery of plasmid DNA from the solution. This can be accomplished by ultrafiltration, precipitation, binding to a particle or surface which binds nucleic acids such as  
20 hydroxyapatite, glass milk or DEAE beads, centrifugation or isobutanol extraction. A preferred method is the use of an ultrafiltration device which works by permeation through a molecular weight selective membrane filter.

25 The method of the invention will now be illustrated by way of the following, non-limiting examples.

#### Example 1

30 A 1.5 ml E. coli culture containing the cloning vector pUC118 was grown overnight to saturation. The E. coli were pelleted and resuspended in 300  $\mu$ l of 100 mM Tris-Cl (tris(hydroxymethyl)aminomethane hydrochloride), 2.5 mM MgCl<sub>2</sub>, 0.5% Triton X-100, pH 8.0. Lysozyme solution, 30  $\mu$ l at 10  $\mu$ g/ $\mu$ l, was added and  
35 incubated at 37°C for 30 minutes. The lysozyme alone did not create microscopically visible ruptures in the

E. coli cells. The E. coli were heated at 95 °C for 5 minutes, and then cooled to 40°C.

Proteinase K solution, 10  $\mu$ l at 10  $\mu$ g/ $\mu$ l, was added and the E. coli were incubated at 50°C for 30 minutes. This enzymatic step resulted in the complete clearing of the solution. Microscopic examination confirmed that no E. coli or fragments were present. Apparently, the lysozyme and heating step had introduced holes in the bacteria allowing the protease to enter and dissolve the cells completely. This lysed solution was again heated at 95°C for 5 minutes, then cooled to 25°C, to inactivate the proteinase K.

The contents of the tube were precipitated by adding 35  $\mu$ l of 3.0 M NaOAc (pH 5.2) + 800  $\mu$ l ethanol, pelleted at 12,000g for 5 minutes, and resuspended in 50  $\mu$ l of TE buffer with ribonuclease (10 mM Tris-Cl, 1 mM EDTA, 20  $\mu$ g/ml pancreatic ribonuclease, pH 8.0). The preparation at this point was analyzed by gel electrophoresis (0.6% agarose in 40mM TrisOAc, 2 mM EDTA, pH 8.3). Following ethidium bromide staining, evaluation of the gel indicated that the lysate consisted largely of fragmented chromosomal DNA, fragmented RNA, and intact plasmid DNA.

To the ribonuclease treated lysate were added 5  $\mu$ l MgCl<sub>2</sub> (100 mM) + 10  $\mu$ l KCl (200 mM) + 0.8  $\mu$ l DTT (100 mM) + 10 units Topoisomerase I (USB) + 80 units T7 Exonuclease (USB). This mixture was incubated for 2 hours at 37°C. Agarose gel electrophoresis of the mixture following incubation showed some degradation of the chromosomal DNA. The RNA smear at the bottom of the gel had been removed. Apparently, T7 exonuclease has RNase activity.

To further improve DNA digestion, 4  $\mu$ l CaCl<sub>2</sub> (100 mM) + 8  $\mu$ l NaCl (3.0 M) + 2.5 units Bal 31 nuclease (USB) were added to the lysate. This mixture was incubated at 30°C for 2 hours. A further improvement

in the purification of the plasmid DNA is observed upon gel electrophoresis of the product, but some additional purification was deemed appropriate.

The remaining chromosomal DNA mandates additional 5 steps to purify the plasmid DNA. To the solution was added 1  $\mu$ l dithiothreitol (DTT) + 8 units Exonuclease III (USB), an enzyme that specifically degrades the ends of double stranded DNA but not plasmid DNA. This mixture was incubated at 37°C for 30 minutes or 60 10 minutes and the products were again evaluated by gel electrophoresis. In each case, a much cleaner preparation of plasmid DNA was obtained. In fact, the two migrating forms of pUC118 plasmid DNA (3.2 kb) could be readily visualized: supercoiled plasmid 15 migrates fast at 2 kb (escaped topoisomerase I conversion) and relaxed, circular (or nicked) plasmid migrates at 3.2 kb. Some remaining DNA was observed, which is probably single stranded DNA, which is 20 resistant to Exonuclease III due to the enzyme's requirement for a double stranded substrate.

At this stage in the experiment, the DNA was precipitated from the sample using 3.0 M NaOAc and ethanol and stored overnight at -20°C. The DNA was collected by centrifugation at 15,000 g for 5 minutes, 25 washed with 0.5 ml 70% ethanol and dissolved in 40  $\mu$ l Exonuclease I buffer (67 mM glycine, 6.7 mM MgCl<sub>2</sub>, 10 mM mercaptoethanol, pH 9.5). Exonuclease I (USB), 25 units, was added and incubated at 37°C for 10 min, 30 min, and 2 hours. Exonuclease I incubation resulted in 30 only a slight improvement in the purity of plasmid DNA, although more improvement might be achieved if more enzyme were used. No degradation of either the supercoiled or relaxed plasmid DNA is observed 35 (compared to pre-exonuclease I control) due to the specificity of Exonuclease I for the ends of single-stranded DNA.

The solution was next heated at 95°C for 5 minutes and cooled to 25°C. The DNA was precipitated using 3.0 M NaOAc and ethanol and redissolved in 21  $\mu$ l of 30 mM NaOAc, 200 mM NaCl, 1 mM ZnCl<sub>2</sub>, pH 4.6. 236 units of S1 nuclease (USB) were then added and the mixture was 5 incubated at 37°C for 1 min, 10 min, 30 min, and 2 hours. The result was a dramatic purification of the plasmid DNA, although some loss of even the plasmid DNA was observed if incubation times of 30 minutes or 10 longer were used. The only band on the gel remaining was relaxed, circular plasmid DNA. This conclusion is supported by the following reasoning: Prior to S1 incubation, the sample was heat denatured at 95°. If the plasmid was either nicked or linearized, the heat 15 would have converted it to single stranded DNA, which would be degraded by S1 nuclease.

Example 2

E. coli containing the plasmid pUC118 was grown by inoculating 500  $\mu$ l of TYGPN both (2.0 g tryptone, 1.0 g yeast extract, 800  $\mu$ l glycerol 0.5 g sodium phosphate and 1.0 g potassium nitrate in 100 ml of deionized water, pH 7.0.) containing 50  $\mu$ g/ml ampicillin. The E. coli was incubated in a sealed 0.5 ml Eppendorf tube at 37°C without agitation or aeration for 10 hours. 20 The cell density before harvesting was about  $1.6 \times 10^9$  cells/ml. The cells were harvested by centrifugation at 12,000g (1 min, 4°C) and resuspended in 300  $\mu$ l of 100 mM Tris-Cl, 2.5 mM MgCl<sub>2</sub>, 0.5% TRITON X-100, pH 8.0. 25 30  $\mu$ l of lysozyme (10 mg/ml) was added to the resuspended cells and the mixture was incubated at 37°C for 30 minutes. The mixture was then heated to 95°C for 5 minutes and then cooled to 50°C.

7.5  $\mu$ l CaCl<sub>2</sub> solution (100 mM) and 10  $\mu$ l proteinase K solution (10mg/ml) was added to the cooled mixture 35 and incubated for 60 minutes at 50°C. The mixture was

then heated to 95°C again for 5 minutes and cooled to 25°.

5 Total DNA was precipitated by addition of 3.0 M NaOAc (pH 5.2) +800  $\mu$ l ethanol and incubating at -20°C for 30 minutes. The precipitated DNA was recovered by centrifugation (12,000g) for 5 minutes at 4°C. The pellet was recovered, washed with 70% ethanol and dried at room temperature.

10 The pellet was then redissolved in 50  $\mu$ l of 50 mM Tris-Cl, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1.0 mM DTT, 20  $\mu$ g/ml RNase (USB), pH 7.5. 30 units of Topoisomerase I (BRL) were then added and the mixture was incubated at 37°C for six hours to degrade RNA and relax supercoiled plasmid DNA. At the end of the 15 incubation time, the mixture was heated to 95°C for 5 minutes to inactivate the enzymes, and then cooled to 25°C.

20 Total DNA was again recovered by precipitation using 4.5  $\mu$ l of 3.0 M NaOAc (pH 5.2) and 110  $\mu$ l of ethanol. After 30 minutes of incubation time at -20°C, the precipitated DNA was collected by centrifugation (12000 g, 5 min. 4°C), washed with 70 % ethanol and dried at room temperature. The resulting material, containing both plasmid and chromosomal DNA was 25 dissolved in 40  $\mu$ l of 30 mM NaOAc, 200 mM NaCl, 1 mM ZnCl<sub>2</sub>, pH 4.6. 118 units of S1 nuclease (USB) were added and the mixture was incubated at 37°C for 60 minutes. The resulting solution was analyzed by agarose gel electrophoresis in 40 mM TrisOAc, 1 mM EDTA, pH 8.3 buffer. The gels showed plasmid DNA of 30 approximately 95% purity.

35 In the foregoing experiment, precipitation/centrifugation was used to recover DNA at the end of two intermediate enzymatic digestion steps to allow for buffer exchange. It will be understood, however, that the use of this particular technique is not essential

to practicing the invention. Thus, ultrafiltration, performed intermittently or continuously, could be used in place of the precipitation/centrifugation steps, thus yielding a method free of centrifugation steps 5 after the initial harvesting of the cells.

Example 3

The experiment of example 2 was repeated except that T4 DNA ligase and AMP was used in place of topoisomerase to relax the plasmid DNA. The DNA pellet 10 was dissolved in 50  $\mu$ l of 50mM Tris-Cl, 10 mM MgCl<sub>2</sub>, 1mM DTE (dithioerythritol), 20  $\mu$ g/ml RNase (USB), pH 7.6. To this 5 units of T4 DNA ligase (BRL) and 2.5  $\mu$ l of 50 mM AMP were added. The mixture was incubated at 37°C for 6 hours and then heated to 95°C for 5 minutes 15 before cooling to 25°C. Comparable purification to Example 2 was achieved.

Example 4

M13 DNA was prepared from a culture of E. coli transformed with M13mp19 replicative form DNA that had 20 been grown in LB broth overnight with agitation. The culture had achieved saturation density.

400  $\mu$ l of the culture were placed in an 0.5 ml Eppendorf tube. 30  $\mu$ l of lysozyme solution (10 mg/ml) was added, and the mixture was incubated at 37°C for 30 minutes. After the incubation, the mixture was heated 25 to 95°C for 5 minutes and then cooled to 50°C.

10  $\mu$ l of Proteinase K solution (10 mg/ml) and 10  $\mu$ l of 100 mM CaCl<sub>2</sub>, were then added and the mixture was incubated at 50°C for 60 minutes, after which it 30 was heated to 95°C for 5 minutes and cooled to 25°C. The nucleic acids in the sample were precipitated by adding 50  $\mu$ l of 3.0 M NaOac (pH 5.2) and 1 ml of ethanol and incubating at -20°C for 45 minutes. The precipitated nucleic acids were collected by 35 centrifugation (12000g, 5 minutes, 4°C), washed with 70% ethanol and dried at room temperature.

5        The dried nucleic acids were dissolved in 75  $\mu$ l of 50 mM Tris-Cl (pH 8.0), 10 mM MgCl<sub>2</sub>, 50 mM NaCl. 20 units of Mbo I (BRL), a restriction enzyme, were added. Following incubation at 37°C for 60 minutes, the mixture was heated to 95°C for 5 minutes and then cooled to 37°C. 20 units of T4 polynucleotide kinase (BRL), 2.5  $\mu$ g RNase and 5  $\mu$ l of 100 mM DTT were then added and the mixture was incubated at 37°C for 60 minutes.

10        As evaluated by agarose gel electrophoresis, this procedure resulted in a significant purification of the single-stranded M13 DNA migrating at about 5000 bp on the gels. Two other bands were visible on the gel, migrating at about 4000 and 6000 bp, which are either 15 alternate sized intracellular forms of the closed circular single-stranded DNA or the double stranded M13 supercoiled and relaxed plasmid DNA. A small amount of single-stranded chromosomal DNA was also observed. This can probably be removed by the addition of a 20 single-stranded exonuclease, such as DNA polymerase, to the enzyme mixture.

Example 5

25        A simple modification of the protocols discussed above makes it possible to use the present invention to isolate RNA from a sample. In this case, RNases are omitted and a mixture of DNases is used to degrade all DNA. Selective membrane filtration of the sample using a 25,000 dalton cut-off membrane isolates total RNA including messenger RNA, transfer RNA and 30 ribosomal RNA.

Example 6

35        The method of the invention can be used to isolate viral particles. Lytic viruses produce viral particles within a host cell which are protected by viral coat proteins. Host DNA, on the other hand is exposed to the surrounding environment upon lysis of the cell.

Viral DNA/RNA can therefore be recovered, separate from host DNA, by the steps of enzymatic lysis of the cell walls or membranes, addition of nucleases to the lysed cells to degrade host nucleic acids, addition of 5 nuclease inhibitors, proteases or heat to inactivate the nucleases and then treatment with a proteolytic enzyme to destroy the nucleases and remove the viral coat protein.

The method of the present invention lends itself 10 readily to automation because it can be performed without resort to centrifugation, phenol extraction, ethanol precipitation or gel electrophoresis. Fig. 1 shows a schematic of a basic instrument for accomplishing this purpose.

15 As shown in Fig. 1, sample containers 2 are held in a temperature control block 1 and supplied with reagents via an automated pipettor 4 controlled by a computer 5. The temperature of the block can be controlled by introducing a circulating liquid (e.g. 20 water) via line 9 to flow through the block and exit via line 10. Liquid flow can be controlled via valve 7 whose operation is controlled to maintain the sample in the holders 2 at the necessary temperatures. Feedstock supplies 6 may conveniently be included within the 25 apparatus housing 3, as may a pump 8 to provide PEG or vacuum to the sample holder as discussed further below. The sample containers may advantageously be microtiter plates having a plurality of wells, the bottom of each of which is in contact through a filtration membrane 30 with a circulating solution of polyethylene glycol (PEG) or a vacuum. (See Fig. 2) The osmotic pressure created by the PEG or the pressure differential caused by the vacuum draws molecules of molecular weight below the cut-off of the membrane out of the sample solution, 35 thus allowing continuous removal of low molecular weight products of proteolytic or nucleolytic digestion.

and facilitating buffer exchanges when needed throughout the process.

Fig. 2 shows schematically a microtiter plate 21 adapted for use in the present invention. Each well 22 of the plate 21 is in contact through an ultrafiltration membrane 24 with a stream of PEG flowing through tube 23. PEG is supplied from vessel 25 into tube 23 and flows through the microtiter plate 21 propelled by pump 8, for example a peristaltic pump.

Fig. 3 shows a cross section of a sample well in the microtiter plate 21 and the adjoining tube 23. PEG solution 31 circulating within tube 23 past ultrafiltration membrane 24 osmotically draws water and materials of molecular weight below the filter's cut-off out of the sample well 22. The height of the impermeable wall 34 defines the final volume of the sample if buffer is not continuously added.

Fig. 4 shows an alternative sample well arrangement in which vacuum rather than osmotic pressure is employed. In this case, each sample well 22 has an ultrafiltration membrane 24 at the bottom of the well. Vacuum is supplied as needed to the bottom side of the membrane to draw water and low molecular weight solutes out of the well while leaving high molecular weight closed circular DNA behind.

Claims

- 1 1. A method for purifying circular DNA from cells or  
2 subcellular organelles of a host organism  
3 containing circular DNA comprising the steps of:  
4 (a) lysing the cells or organelles to release  
5 circular DNA;  
6 (b) enzymatically treating the preparation  
7 containing released circular DNA with a protease  
8 enzyme;  
9 (c) heating the protease-treated preparation  
10 to denature substantially all non-circular DNA to  
11 single stranded form;  
12 (d) treating the heat treated preparation  
13 with nuclease enzymes effective to selectively  
14 digest RNA and chromosomal DNA while leaving  
15 closed circular DNA intact; and  
16 (e) recovering closed circular DNA.
- 1 2. A method according to claim 1, wherein the host  
2 organism is E. coli and the protease enzyme is  
3 Proteinase K.
- 1 3. A method according to claim 1, wherein the  
2 circular DNA is single stranded and wherein the  
3 nuclease enzymes are selected from among nucleases  
4 substantially free from single-stranded  
5 endonuclease activity.
- 1 4. A method according to claim 1, wherein the  
2 circular DNA is double stranded, further  
3 comprising the step of treating the preparation to  
4 relax supercoiled plasmid DNA prior to treatment  
5 with the nuclease enzymes.

- 1 5. A method according to claim 4, wherein the  
2 preparation is treated with topoisomerase to relax  
3 supercoiled DNA.
- 1 6. A method according to claim 4, wherein the  
2 nuclease enzymes are selected from the group  
3 consisting of Mung Bean Nuclease, S1 Nuclease, P1  
4 Nuclease, T7 exonuclease, Bal 31 Nuclease,  
5 Exonuclease I, Exonuclease III, Exonuclease VII  
6 and Lambda Exonuclease.
- 1 7. A method according to claim 1, wherein the  
2 nuclease enzymes are selected from the group  
3 consisting of Mung Bean Nuclease, S1 Nuclease, P1  
4 Nuclease, T7 exonuclease, Bal 31 Nuclease,  
5 Exonucleases I, Exonuclease III, Exonuclease VII  
6 and Lambda Exonuclease.
- 1 8. A method according to claim 7, wherein the host  
2 organism is E. coli and the protease enzyme is  
3 Proteinase K.
- 1 9. A method of purifying viral particles from  
2 infected host cells comprising the steps of
  - 3 (a) lysing the host cells to release viral  
4 particles and host nucleic acids from the cells;
  - 5 (b) adding nucleases to the lysed cells to  
6 degrade host nucleic acids;
  - 7 (c) adding nuclease inhibitors or  
8 inactivators to the nuclease-treated lysed cells;
  - 9 (d) treating the preparation with a  
10 proteolytic enzyme to destroy the nucleases and  
11 remove coat protein from the viral particles; and
  - 12 (e) recovery to uncoated viral particles.

1 10. A method for separating and recovering closed  
2 circular DNA from a composition containing closed  
3 circular DNA and linear DNA comprising the steps  
4 of

5 (a) treating the composition to denature  
6 substantially all of the linear DNA to single-  
7 stranded form;

8 (b) treating the denatured composition with  
9 nuclease enzymes effective to selectively digest  
10 linear DNA while leaving closed circular DNA  
11 intact; and

12 (c) recovering the closed circular DNA.

1 11. A method according to claim 10, wherein the  
2 circular DNA is single-stranded and wherein the  
3 nuclease enzymes are selected from among nucleases  
4 substantially free from single-stranded  
5 endonuclease activity.

1 12. A method according to claim 10, wherein the  
2 circular DNA is double-stranded, further  
3 comprising the step of treating the composition to  
4 relax supercoiled plasmid DNA prior to treatment  
5 with the nuclease enzymes.

1 13. A method according to claim 12, wherein the  
2 composition is treated with topoisomerase to relax  
3 supercoiled DNA.

1 14. A method according to claim 12, wherein the  
2 nuclease enzymes are selected from the group  
3 consisting of Mung Bean Nuclease, S1 Nuclease, P1  
4 Nuclease, T7 exonuclease, Bal 31 Nuclease,  
5 Exonuclease I, Exonuclease III, Exonuclease VII  
6 and Lambda Exonuclease.

1 15. A method according to claim, 10, wherein the  
2 nuclease enzymes are selected from the group  
3 consisting of Mung Bean Nuclease, S1 Nuclease, P1  
4 Nuclease, T7 exonuclease, Bal 31 Nuclease,  
5 Exonuclease I, Exonuclease III, Exonuclease VII  
6 and Lambda Exonuclease.

1 16. A method according to claim 10, further comprising  
2 the step of treating the composition with a  
3 restriction endonuclease prior to the denaturation  
4 step, wherein the restriction endonuclease is  
5 selected such that the circular DNA to be  
6 separated and recovered is not cleaved by the  
7 restriction endonuclease.

1 17. An apparatus for separation and recovery of closed  
2 circular DNA, comprising  
3 (a) a sample holder having a plurality of  
4 sample wells, each of said wells having an  
5 ultrafiltration membrane disposed in a lower  
6 portion thereof through which liquid can be  
7 removed from the well;  
8 (b) means for controlling the temperature of  
9 the sample holder and of material within the  
10 wells;  
11 (c) means for drawing liquid through the  
12 ultrafiltration membranes at the bottom of each  
13 well; and  
14 (d) means for adding reagents to each of  
15 said sample wells.

1 18. An apparatus according to claim 17, wherein the  
2 means for drawing liquid through the  
3 ultrafiltration membranes is a vacuum pump.

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1       19. An apparatus according to claim 17, wherein the  
2       means for drawing liquid through the  
3       ultrafiltration membranes is a manifold containing  
4       a circulating fluid which causes water to pass  
5       through the ultrafiltration membrane as a result  
6       of osmotic pressure.

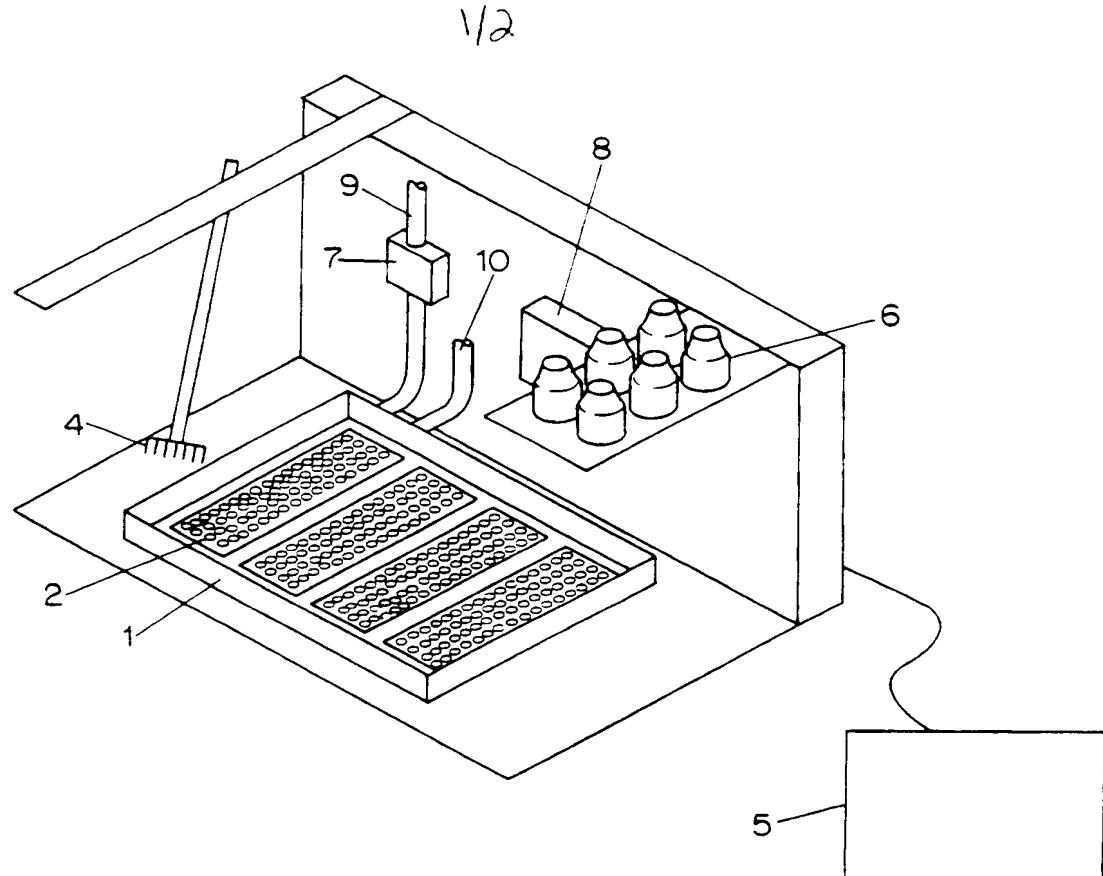


FIG. 1

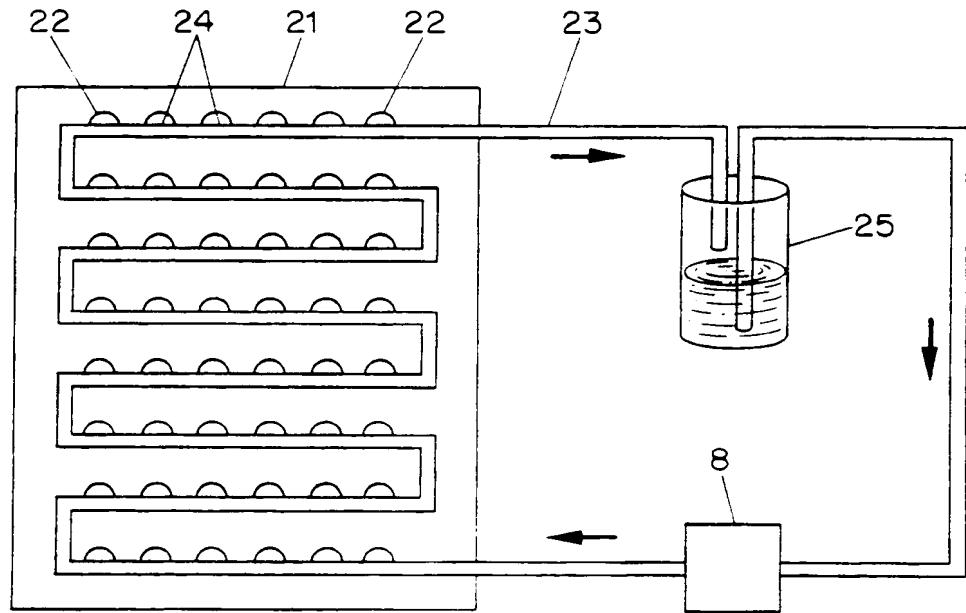


FIG. 2

SUBSTITUTE SHEET

2/2

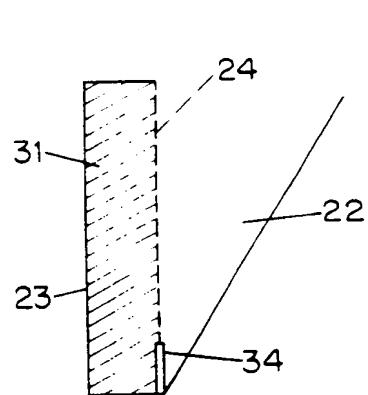


FIG. 3

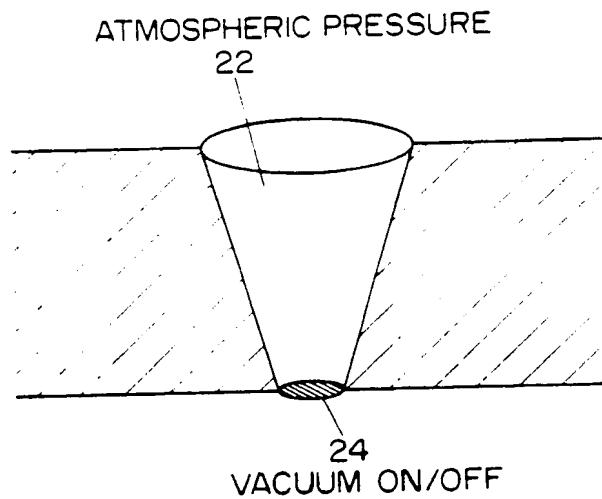


FIG. 4

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00540

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 19/34; C12N 7/02; C12M 1/12  
US CL : 435/91, 239, 311

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>4</sup>

Classification System	Classification Symbols
U.S.	435/91, 239, 311

Documentation Searched other than Minimum Documentation  
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APS, Biosis, World Patent Index

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	Maniatis et al., "Molecular Cloning: A Laboratory Manual", published 1982 by Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.), see pages 80-87 and 89-91.	1-16
Y	Ausubel et al., "Current Protocols in Molecular Biology", published 1989 by John Wiley (New York), see pages 3.11.1-3.11.4 and 3.12.1-3.12.3.	1-16
Y	US, A, 5,047,215 (Manns), 10 September 1991, see entire document	17-19
Y	Atkins, "Physical Chemistry", published 1978 by W. H. Freeman and Company (San Francisco), see pages 222-225.	17-19

### \* Special categories of cited documents:<sup>16</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
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## IV. CERTIFICATION

Date of the Actual Completion of the International Search<sup>2</sup>

27 APRIL 1992

Date of Mailing of this International Search Report<sup>2</sup>

06 MAY 1992

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*Richard Frese Jr.*  
Philip W. Carter



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> <b>PCT/GB87/00384</b>	<b>(74) Agent:</b> <b>REDDIE &amp; GROSE; 16 Theobalds Road, London WC1X 8PL (GB).</b>
<b>(22) International Filing Date:</b> <b>3 June 1987 (03.06.87)</b>	
<b>(31) Priority Application Number:</b> <b>8613476</b>	<b>(81) Designated States:</b> <b>AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</b>
<b>(32) Priority Date:</b> <b>4 June 1986 (04.06.86)</b>	
<b>(33) Priority Country:</b> <b>GB</b>	

**(71) Applicant (for all designated States except US):** **THE LONDON HOSPITAL MEDICAL COLLEGE [GB/GB]; Turner Street, London E1 (GB).**

**(72) Inventors; and**  
**(75) Inventors/Applicants (for US only):** **COATES, Anthony, Robert, Milnes [GB/GB]; Hereford Cottage, 135 Gloucester Road, London SW7 (GB). HALL, Lucinda, Mary, Clare [GB/GB]; 38, Lodge Drive, Palmers Green, London N13 5JZ (GB). BLENCH, Ian [GB/GB]; 77 The Woodlands, Upper Norwood, London SE19 3EH (GB).**

**Published**

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*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

**(54) Title:** **A METHOD OF SEQUENCING NUCLEIC ACIDS**

**(57) Abstract**

A method of sequencing nucleic acids, such as DNA, which eliminates centrifugation steps during pre-sequencing purification, reduces manipulation of extracted products and is suitable for automation, resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane. The filter membrane may retain nucleic acids by size exclusion and/or adsorb nucleic acids by surface binding.

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## A method of sequencing nucleic acids.

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The present invention relates to an improved method of sequencing nucleic acids, and particularly but not exclusively to an improved method of sequencing DNA.

The technique of nucleotide sequence analysis is of central importance in molecular biology and biotechnology. Contemporary methods of sequencing are mostly based on the high resolving power of polyacrylamide-gel electrophoresis. By this technique two oligonucleotides which differ in size by only a single nucleotide residue can be resolved by virtue of their differing relative interactions with the gel matrix. Alternative resolving methods are currently being investigated, for example column based systems such as high pressure liquid chromatography (HPLC).

In order to determine the sequence of nucleotides in a nucleic acid such as DNA a series of fragments are produced with one end in common and the other varying in position along the chain. A minimum of four types of series of fragments is produced, each group being terminated by or cleaved at one of the four possible bases by specific chemical or enzymic means.

There are two well known ways in which DNA fragments ending in or cleaved at each of the four bases are produced for sequence analysis. In the method described by Maxam and Gilbert (reference 1 and 2) single- or double-stranded DNA molecules are labelled with radioactive  $^{32}\text{P}$  phosphate at a unique 5' or 3' terminus and the nucleotide chains are chemically modified at specific bases and then cleaved by alkaline or piperidine hydrolysis. The chain termination method of Sanger et al (reference 3 to 6) involves enzymic copying of single-stranded DNA fragments using a DNA polymerase to transcribe specific regions of the chain under controlled conditions.

In both the above described methods it is necessary to extract and purify the nucleic acids prior to initiating sequencing reactions. Conventionally RNA and DNA from tissues, cells, plasmids and viruses are extracted and purified by lysis or digestion of the protein coat (e.g. with a protease - such as proteinase K - which is able to digest the protein coat without degrading the nucleic acids) followed by extraction with solvent, such as phenol-chloroform, and precipitation with e.g. ethanol. Separation of nucleic acids from suspension in solvents is achieved by repeated centrifugation.

The phenol-chloroform reagent is a toxic corrosive liquid which is unpleasant to handle. After extraction with phenol-chloroform it is necessary to separate the aqueous from the organic phase. Centrifugation steps in the extraction/purification procedures are time consuming and tedious when carried out manually, and are difficult to automate satisfactorily.

The present invention provides an improved method of sequencing nucleic acids, such as DNA, which eliminates centrifugation steps during pre-sequencing purification, reduces manipulation of extracted products and is suitable for automation.

According to the invention, the improvement resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane.

The filter membrane may (a) retain nucleic acids by size exclusion and/or may (b) adsorb nucleic acids by surface binding. Examples of filter membranes of type (a) are anisotropic low adsorptive ultrafiltration membranes such as the type-YM membranes marketed by Amicon Corporation (US Patent No. 3488768). Examples of filter

membranes of type (b) are nitrocellulose membranes, NA45(TM), DE81(TM), Genescreen (TM), Hybond (TM) and the like; filter membranes having non-specific binding sites may be partially blocked with Bovine Serum Albumin (BSA) or other inert material.

An advantage of using ultrafiltration for separation and purification of nucleic acids is that the material collected on the membrane can be further treated *in situ*. For example, chemical or enzymic reagents can be applied to the membrane surface after filtration in order to prepare the trapped nucleic acids for sequencing.

The method of the invention can be used in an improved enzymic sequencing process including the following steps:-

- 1] A vector carrying a DNA insert to be sequenced is cultured in a host,
- 2] vector particles containing the DNA inserts are separated from the host by filtration,
- 3] a protease is applied which will digest the protein coat of the vector without degrading the DNA,
- 4] the DNA is purified and concentrated by pressure activated ultrafiltration through a membrane which retains the DNA,
- 5] primer is added and annealed to the extracted DNA on the membrane,
- 6] a polymerisation enzyme together with a suitable nucleotide mixture is applied to the DNA on the membrane, the enzyme being one which is capable of catalysing the faithful incorporation of nucleotides onto a primed template,

7] chase nucleotides are added if necessary in order to complete the polymerisation reaction,

8] the reaction is terminated and the newly synthesized DNA is disassociated from the template, and

9] a sample of DNA is loaded onto a polyacrylamide gel for electrophoresis, or onto an alternative separation system.

In the above described method any host/vector system can be used in which vector DNA is released from the host cell, generally in the form of a protein coated particle. The DNA could be single or double stranded; if the DNA is double stranded a denaturation step must be included before annealing in order to separate the strands. The chosen vector must have been manipulated so as to carry both the DNA fragment to be sequenced and the appropriate primer hybridisation site. A particularly suitable host is the bacterium E.Coli, and suitable vectors are bacteriophage M13, plasmid pEMBL and F1 derived vectors.

Examples of suitable protease are chymotrypsin, elastase, subtilisin and thermolysin. Residual protease activity can be terminated after the reaction has proceeded to completion by use of an ethanol wash or a protease inhibitor. Alternatively, the protease may have autolytic activity and hence be self-terminating. The catalysis enzyme is preferably a DNA polymerase such as a Klenow fragment of DNA polymerase I or reverse transcriptase. The enzyme must not have exonuclease activity which would digest the primer; if present, exonuclease activity can be blocked by the addition of a blocking group at the 5' end of the molecule.

If the above described method were adapted for sequencing with ribonucleotides, then ribonucleotides would be used instead of deoxyribonucleotides in the polymerisation step. In such a case an appropriate RNA polymerase would be used, possibly without the need

for a primer. Alternatively, if a suitable vector for RNA were developed, RNA could be used as the template.

One embodiment of the invention will now be described in detail, by way of example only. The Example illustrates an improved method of DNA sequencing using the chain termination method described by Sanger.

#### EXAMPLE

The method comprises the following steps :

- 1) Bacteriophage M13 with a DNA insert is cultured overnight in E.Coli in suitable culture medium.
- 2) The bacteria are separated from the phage by filtration with a 0.22 micron membrane, such as hydrophilic Durapore (Millipore). The bacteria remain on the filter and the DNA-carrying phage pass through the membrane into a collection vessel.
- 3) The phage are incubated with a protease, such as chymotrypsin, in order to digest the protein coat.
- 4) The DNA from the phage is separated from the digested protein by pressure activated ultrafiltration through a membrane which retains the DNA but not the digested protein. A preferred size-exclusion membrane is YM10 (Amicon) which retains globular molecules of size greater than 10 kDaltons. Alternatively, an adsorptive membrane such as nitrocellulose may be used. Non-specific binding of reagents to the filter can be prevented by the addition of 1 % bovine serum albumin.
- 5) The primer is added as a droplet or a spray to the DNA on the filter membrane and is annealed for 20 minutes at 55 to 65°C to allow specific hybridisation.

- 6 -

- 6) Premixed nucleotides and DNA polymerase I Klenow fragment are added as a droplet or a spray to the filter membrane and are incubated at 20°C for 15 minutes. The nucleotides are incorporated onto the primed template to synthesize DNA by chain extension. The nucleotides may include labelled nucleotides and chain terminating nucleotides.
- 7) Chase nucleotides are added if necessary.
- 8) A formamide droplet or spray is placed on the membrane and heated at 90°C for 5 minutes in order to terminate reaction and caused disassociation of newly synthesized DNA from the template.
- 9) A DNA sample is loaded onto a conventional polyacrylamide gel.
- 10) The DNA fragments are separated by electrophoresis.

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1. Maxam, A.M. and Gilbert, W (1977), Proc.Natl. Acad.Sci., USA., 74, 560-564.
2. Maxam, A.M. and Gilbert, W (1980), in Methods in Enzymology, (ed Wil, R), 68, p.499, Academic Press, London and New York.
3. Sanger, F. and Coulson, A.R. (1978), FEBS lett., 87, 107-110.
4. Sanger, F. and Coulson, A.R. (1975), J.Mol.Biol., 94, 441-448.
5. Sanger, F., Nicklen, S. and Coulson, A.R. (1977), Proc.Natl.Acad.Sci., USA, 74, 5463-5467.
6. Air, G.M., Sanger, F. and Coulson, A.R. (1976), J.Mol.Biol., 108, 519-533.

CLAIMS :

1. An improved method of sequencing nucleic acids wherein the improvement resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane.

2. An improved enzymic sequencing process for DNA which includes the following steps :

- i] a vector carrying a DNA insert to be sequenced is cultured in a host,
- ii] vector particles containing the DNA inserts are separated from the host by filtration,
- iii] a protease is applied which will digest the protein coat of the vector without degrading the DNA,
- iv] the DNA is purified and concentrated by pressure activated ultrafiltration through a membrane which retains the DNA,
- v] primer is added and annealed to the extracted DNA on the membrane,
- vi] a polymerisation enzyme together with a suitable nucleotide mixture is applied to the DNA on the membrane, the enzyme being one which is capable of catalysing the faithful incorporation of nucleotides onto a primed template,
- vii] chase nucleotides are added if necessary in order to complete the polymerisation reaction,

viii] the reaction is terminated and the newly synthesized DNA is disassociated from the template, and

ix] a sample of DNA is loaded onto a polyacrylamide gel for electrophoresis, or onto an alternative separation system.

3. An improved method of DNA sequencing using the chain termination method of Sanger, which includes the following steps :

a) Bacterophage M13 with a DNA insert is cultured in E.Coli in suitable culture medium,

b) the bacteria are separated from the phage by filtration with a hydrophilic membrane,

c) the phage are incubated with chymotrypsin protease,

d) the DNA from the phage is purified and concentrated by pressure activated ultrafiltration through a membrane which retains the DNA (e.g. a size-exclusion membrane which retains globular molecules of size greater than 10 kDaltons or an adsorptive membrane such as nitrocellulose),

e) primer is added as a droplet or a spray to the DNA on the filter membrane and is annealed for 20 minutes at 55 to 65°C,

f) premixed nucleotides and DNA polymerase I Klenow fragment are added as a droplet or a spray to the filter membrane and are incubated at 20°C for 15 minutes,

g) chase nucleotides are added if necessary,

h) a formamide droplet or spray is placed on the membrane and heated at 90°C for 5 minutes,

- 9 -

i) a DNA sample is loaded onto a polyacrylamide gel, and

j) DNA fragments are separated by electrophoresis.

4. An improved method of sequencing, substantially as hereinbefore described.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 87/00384

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all):

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 12 Q 1/68

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
IPC <sup>4</sup>	C 12 Q 1/00
	B 01 D 15/00

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ***	Relevant to Claim No. **
X	EP, A, 0121778 (HOECHST) 17 October 1984 see page 3, line 8 - page 4, line 24; page 5, lines 1-6 --	1-4
X	EP, A, 0127737 (GAMBRO LUNDIA) 12 December 1984 see page 1, lines 17-26; page 2, lines 10-15; page 8, lines 26-31 --	1-4
X	Chemical Abstracts, volume 79, 15 October 1973, (Columbus, Ohio, US), C.W. Hancher et al.: "Evaluation of ultrafiltration membranes with biological macromolecules", see page 165, abstract 88983n, & Biotechnol. Bioeng. 1973, 15(4), 677-91 -----	1-4

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## IV. CERTIFICATION

Date of the Actual Completion of the International Search

10th September 1987

Date of Mailing of this International Search Report

19 OCT 1987

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

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EP-A- 0121778	17/10/84	DE-A- 3308932 JP-A- 59173094 US-A- 4623723	13/09/84 29/09/84 18/11/86
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 4 :</b>  <b>C12Q 1/68</b>		<b>(11) International Publication Number:</b> <b>WO 87/ 07645</b>  <b>(43) International Publication Date:</b> <b>17 December 1987 (17.12.87)</b>
<b>(21) International Application Number:</b> <b>PCT/GB87/00384</b> <b>(22) International Filing Date:</b> <b>3 June 1987 (03.06.87)</b>  <b>(31) Priority Application Number:</b> <b>8613476</b> <b>(32) Priority Date:</b> <b>4 June 1986 (04.06.86)</b> <b>(33) Priority Country:</b> <b>GB</b>		<b>(74) Agent:</b> <b>REDDIE &amp; GROSE; 16 Theobalds Road, London WC1X 8PL (GB).</b>  <b>(81) Designated States:</b> <b>AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</b>
<b>(71) Applicant (for all designated States except US):</b> <b>THE LONDON HOSPITAL MEDICAL COLLEGE [GB/GB]; Turner Street, London E1 (GB).</b>  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> <b>COATES, Anthony, Robert, Milnes [GB/GB]; Hereford Cottage, 135 Gloucester Road, London SW7 (GB). HALL, Lucinda, Mary, Clare [GB/GB]; 38, Lodge Drive, Palmers Green, London N13 5JZ (GB). BLENCH, Ian [GB/GB]; 77 The Woodlands, Upper Norwood, London SE19 3EH (GB).</b>		<b>Published</b> <i>With international search report</i> <i>With amended claims.</i>  <b>Date of publication of the amended claims:</b> <b>28 January 1988 (28.01.88)</b>
<b>(54) Title:</b> <b>A METHOD OF SEQUENCING NUCLEIC ACIDS</b>		
<b>(57) Abstract</b>  A method of sequencing nucleic acids, such as DNA, which eliminates centrifugation steps during pre-sequencing purification, reduces manipulation of extracted products and is suitable for automation, resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane. The filter membrane may retain nucleic acids by size exclusion and/or adsorb nucleic acids by surface binding.		

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## AMENDED CLAIMS

[received by the International Bureau on 21 December 1987 (21.12.87);  
original claim 1 amended; remaining claims unchanged (1 page)]

1. A method for the sequencing of nucleic acid comprising recovering, from a host/vector system, vector particles containing a nucleic acid insert to be sequenced, treating the vector particles with a protease to digest proteinaceous contaminants, removing digestion products by ultrafiltration through a membrane which retains purified nucleic acid, and, thereafter processing the purified nucleic acid without removal from the membrane to produce a series of nucleic acid fragments for subsequent separation by electrophoresis.



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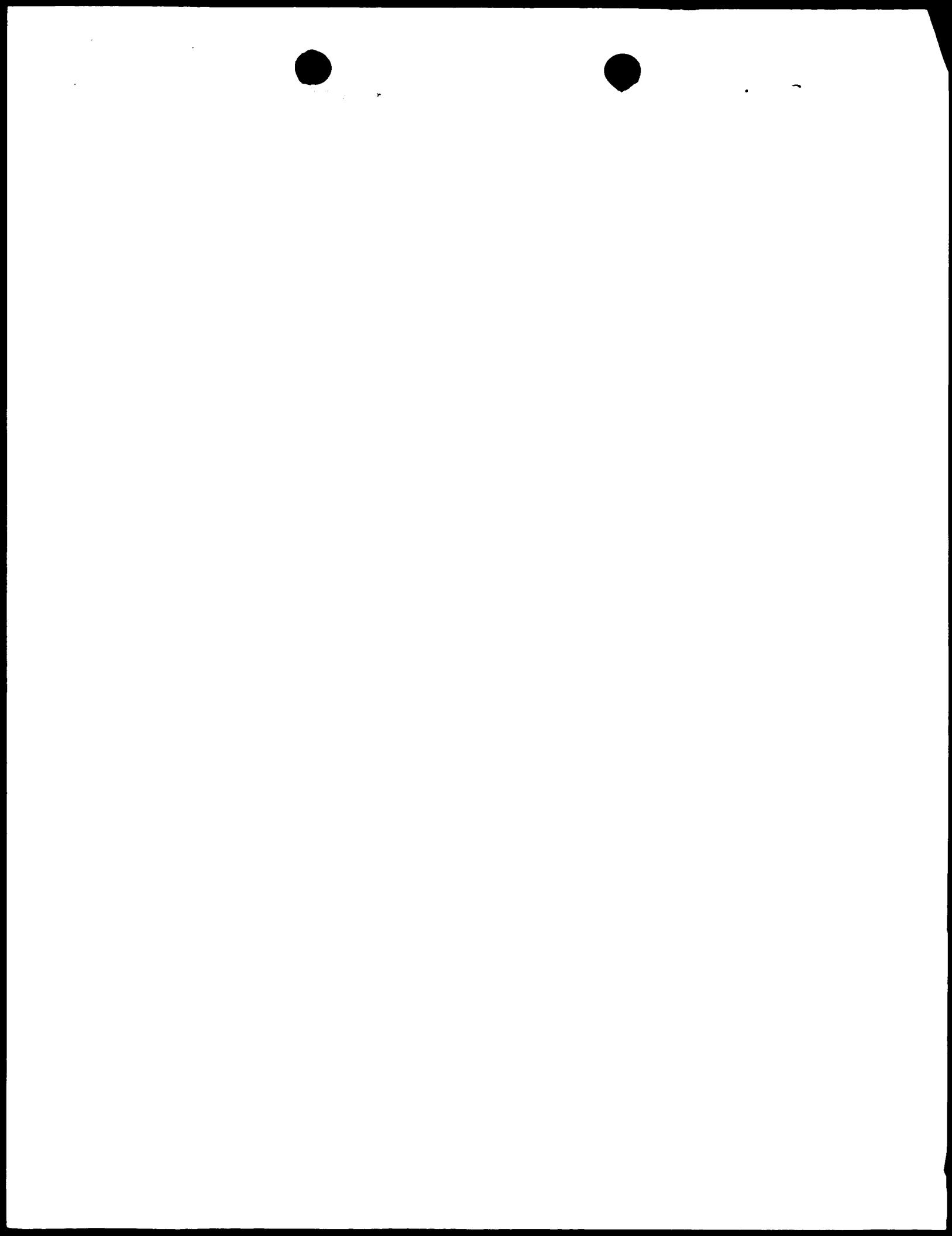
09/93668  
531 Rec'd PCT 13 SEP 2001  
MCA-44897

Date: 12/09/2000

MILLIPORE CORPORATION  
Attn. Hubbard, John Dana  
80 Ashby Road  
Bedford, Massachusetts 01730  
UNITED STATES OF AMERICA

Docketed By:	100 9-14-00
Due Date:	Act 19-10-12
Reminder (s)	11-12
Action Due:	100 11-12
In Database	100 11-12

12-12



## PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:  
**MILLIPORE CORPORATION**  
 Attn. Hubbard, John Dana  
 80 Ashby Road  
 Bedford, Massachusetts 01730  
 UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT  
OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing (day/month/year)	12/09/2000
Applicant's or agent's file reference <b>MCA-448A PC</b>	<b>FOR FURTHER ACTION</b>	See paragraphs 1 and 4 below
International application No. <b>PCT/US 00/ 11926</b>	International filing date (day/month/year)	02/05/2000
Applicant <b>MILLIPORE CORPORATION.</b>		

1.  The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
 34, chemin des Colombettes  
 1211 Geneva 20, Switzerland  
 Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2.  The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3.  **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

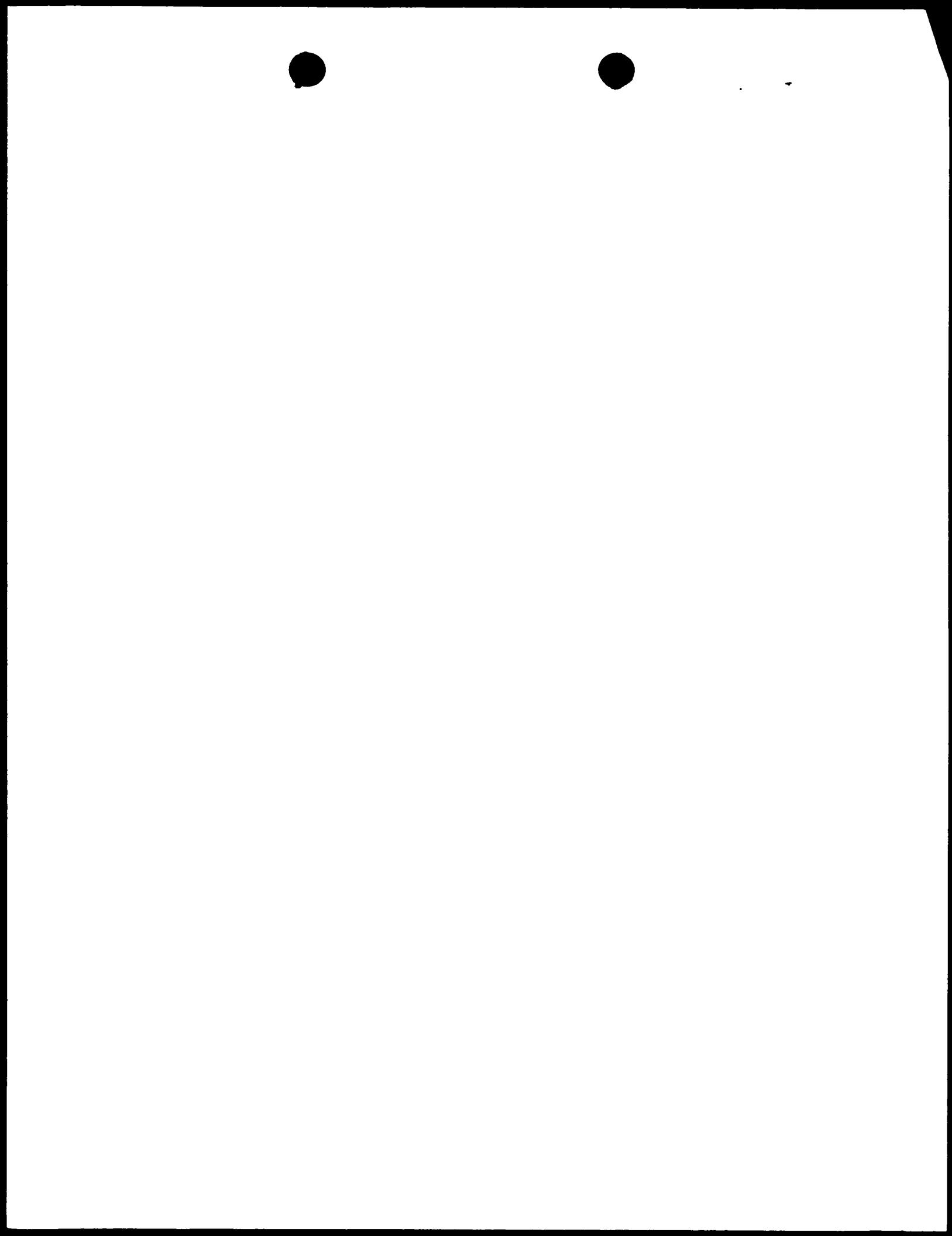
Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority

European Patent Office, P.B. 5818 Patentlaan 2  
 NL-2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

**Andria Overbeeke-Siepkes**



## NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

### INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

#### What parts of the International application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

#### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

#### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

#### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

**The amendments must be made in the language in which the international application is to be published.**

#### What documents must/may accompany the amendments?

##### Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

**The letter must be in English or French, at the choice of the applicant. However, if the language of the International application is English, the letter must be in English; if the language of the International application is French, the letter must be in French.**



## NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

**The following examples illustrate the manner in which amendments must be explained in the accompanying letter:**

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

### **"Statement under article 19(1)" (Rule 46.4)**

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international application is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

### **Consequence if a demand for international preliminary examination has already been filed**

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

### **Consequence with regard to translation of the international application for entry into the national phase**

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

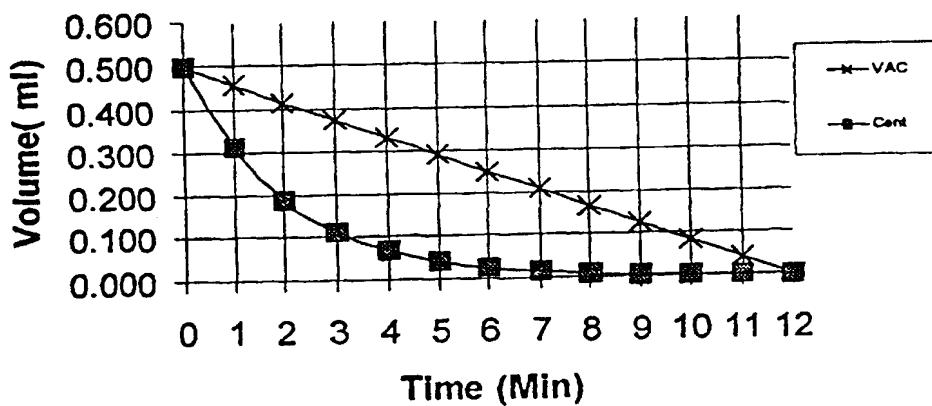




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12N 15/10, C07K 1/34, B01D 61/14</b>		A1	(11) International Publication Number: <b>WO 00/66723</b>
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(21) International Application Number: PCT/US00/11926		(81) Designated States: JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 2 May 2000 (02.05.00)			
(30) Priority Data: 60/132,369 4 May 1999 (04.05.99) US 60/182,357 14 February 2000 (14.02.00) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/182,357 (CON) Filed on 14 February 2000 (14.02.00)			
(71) Applicant (for all designated States except US): MILLIPORE CORPORATION [US/US]; 80 Ashby Road, Bedford, MA 01730 (US).			
(72) Inventor; and (75) Inventor/Applicant (for US only): LEONARD, Jack, T. [US/US]; 19 Ricker Circle, South Hamilton, MA 01982 (US).			
(74) Agent: HUBBARD, John, Dana; Millipore Corporation, 80 Ashby Road, Bedford, MA 01730 (US).			

(54) Title: METHOD OF ULTRAFILTRATION

**UF Throughput**(Vac.=12psi, Cent.=2000g, Flux=.016ml/min/cm<sup>2</sup>/psi, Area=.034 in<sup>2</sup>)

## (57) Abstract

A process for ultrafiltration using constant pressure differential as the driving force is disclosed. This process is particularly suited for use in concentrating or purifying proteins and/or nucleic acids, often without any need for one or more diafiltration steps. The process is particularly suited for small volume applications, such as small concentrator devices and multiple well plates that typically use starting volumes of liquids of less than about 500 microliters. The steps include adding a liquid volume above an ultrafiltration membrane and applying a constant pressure differential at a force and length of time to achieve the desired concentration on the upstream side of the membrane. The concentrate is then diluted or removed for further processing.

